

Validation of a whole-body cortisol extraction procedure for channel catfish (*Ictalurus punctatus*) fry

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Abstract We validated a whole-body cortisol extraction technique for channel catfish, *Ictalurus punctatus*, fry. Three volume enhancement methods were tested: CAL method (zero calibrator A diluent added to lipid extract), PBS method (phosphate buffered saline added to lipid extract), and VO method (food grade vegetable oil added to lipid extract). The volume enhancement extracts were evaluated using a commercial radioimmunoassay kit. Sensitivity, accuracy, precision, reproducibility, and parallelism could not be determined for the PBS method as cortisol levels were not detected in any of the extracted samples. Intra-assay coefficient of variation (CV) for the CAL and VO methods were 7.3 and 8.3%, respectively, while inter-assay CV were 9.6 and 10.6%, respectively. Based on the sensitivity, accuracy, precision, reproducibility, and parallelism results, we conclude that the CAL method is the most appropriate method for volume enhancement of catfish fry lipid extract. Using the CAL method to detect cortisol in catfish fry, fish were stressed daily for 2 weeks. Fry weights were similar throughout the study while whole-body cortisol levels

were higher ($P < 0.01$) in stressed fish after 1 day of stress. These data show the CAL method can effectively measure whole-body cortisol in catfish fry.

Keywords Whole-body cortisol · Cortisol extraction · Catfish fry

Introduction

Stress has been defined as a physiological cascade of events that occurs when an individual attempts to re-establish homeostatic norms in the face of a perceived threat (Schreck et al. 2001). Common to catfish fry production, high stocking densities and intensive handling during transport can result in fish being exposed to high ammonia and low dissolved oxygen levels (Abdalla and Romaire 1996; Torrants et al. 2003), both of which result in fish stress. The most common method of determining the stress response in fish is to measure changes in circulating levels of cortisol (Strange 1980; Donaldson 1981; Tomasso et al. 1981; Wise et al. 1993). The most common method of quantifying blood concentrations of cortisol is with the use of a radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA). These assays can accurately quantify cortisol concentrations in individual fish but typically

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require 20–200 µl of plasma. It is difficult if not impossible to obtain this volume of plasma from catfish fry. Plasma samples could be pooled from multiple fish but pooling of samples can lead to the inability to detect individual responses to treatments.

A probable alternative to pooling samples from small fish is to extract cortisol from the whole body of the fish. Whole-body cortisol assays of larval rainbow trout (*Oncorhynchus mykiss*) and yellow perch (*Perca flavescens*) (Barry et al. 1995; Jentoft et al. 2003), juvenile summer flounder (*Paralichthys dentatus*) and winter flounder (*Pseudopleuronectes americanus*) (Hinkle and Specker 2003; Breves and Specker 2002), and zebra fish (*Danio rerio*) and golden shiners (*Notemigonus crysoleucas*) (Ramsay et al. 2006; Sink et al. 2007) have been published. The methodology of the extraction procedures varies in these papers, the description of the methods used is sometimes incomplete, and the validation of the cortisol assay is not always complete.

The effects of stress on catfish fry are not known and an evaluation of stress responses may lead to improved management practices that minimize stress and improve production efficiency. The objective of this paper was to validate a whole-body cortisol extraction procedure for use in measuring cortisol concentrations in catfish fry. This technique was then further tested in catfish fry that were exercised for 10 min daily for 2 weeks.

Materials and methods

Fish and animal-care procedures

NWAC 103-line channel catfish (male and female) fry from three separate families were hatched and maintained in 40-gallon fiberglass tanks with a single-pass flow-rate of 8 l/min with aeration, at approximately 27°C, and on a 14:10 light:dark photoperiod. Fish were fed once daily to satiety using a commercial floating catfish feed (36% crude protein; Land O'Lakes Farmland Feed, Fort Dodge, IA). All animal procedures followed accepted standards of animal care, approved by the Institutional Animal Care and Use Committee (IACUC) according to United States Department of Agriculture, Agricultural Research Service policies and procedures.

Whole-body cortisol measurement

Three protocols were evaluated for use in extracting cortisol in channel catfish fry. In the first method, the lipid extract was reconstituted with 100 µl of zero calibrator A (Siemens Medical Diagnostics, Los Angeles, CA) (CAL method) diluent supplied with the cortisol RIA kit. The zero calibrator A buffer was the same buffer used to make the standard curve. In the second method, the lipid extract was reconstituted with 1 ml of BupH™ Modified Dulbecco's Phosphate Buffered Saline (Pierce, Rockford, IL) (PBS method) to increase volume of the sample as described by Ramsay et al. (2006) and Sink et al. (2007). In the third method, 100 µl of food-grade vegetable oil (Enova™ oil, Kao Health and Nutrition, Itasca, IL) (VO method) per gram of body weight was added to increase the volume of the extract before adding the homogenized sample and ethyl ether, as described in Sink et al. (2007).

Catfish fry were dried with a paper towel and weighed to the nearest 0.1 g prior to extraction. Cortisol extraction was performed using the method described by Sink et al. (2007), with some modifications. Briefly, the samples, still in the storage tube, were homogenized in 1 ml of PBS, using a Qiagen Tissue Lyser for 15 min. Following homogenization, the sample was transferred to a 15-ml test tube and an additional 1 ml of PBS was used to rinse any remaining sample from the 2-ml tube. The addition of PBS for homogenizing and rinsing was done to all three treatments. Aliquots of 5 ml of laboratory grade ether were added to each sample and vortexed for 1 min. The samples were then centrifuged for 10 min at 3,000 rpm and frozen immediately at –20°C for 2 h. The unfrozen portion was then transferred to a fresh 15-ml tube and the ethyl ether evaporated under nitrogen. The remaining lipid extract was then stored at –20°C until ready for radioimmunoassay (RIA) analysis. Zero calibrator A diluent, PBS, or VO were added to the extracts prior to RIA.

Whole-body cortisol was measured in individual samples of tissue extract using a commercially available Coat-A-Count cortisol kit (Siemens Medical Diagnostics) that had been previously validated for use to measure plasma levels of cortisol in channel catfish (Davis et al. 1993). Extracts from all three extraction procedures were used for validation. Precision and reproducibility of the assay (intra- and

inter-assay variation), were determined from separate samples of pooled extract ($N = 5$) catfish fry having elevated cortisol levels and calculated as coefficients of variation (CV). Fish were pooled together to ensure we had enough extract to complete the validation. Precision was tested by comparing results of serial dilutions (0, 25, 50, and 75%) made by adding zero calibrator A diluent to the extract. The accuracy of the assay was determined as the percent recovery of cortisol standard added at varying levels to catfish (not stressed) extract. Assay sensitivity was calculated as the amount of cortisol equal to B_0 (value of the zero standard) minus two standard deviations when interpolated from the standard curve. Whole-body cortisol was calculated as ng/g of body weight of catfish fry tissue.

Physical stress and sample collection

In order to determine cortisol responses in catfish fry to physical stress, 3-week-old channel catfish fry from three families were pooled and stocked at 200 fish per tank in 76-l aquaria. Nine aquaria of fish were assigned as exercise group, whereas the other nine were assigned as control. Fish were fed daily. Fish in the exercise aquaria were exposed to stress daily (10 min) through lowering of the water level and vigorous chasing with a net. In contrast, fish in the control aquaria were undisturbed. Whole-body cortisol measurements were taken at day 0, 7, and 14 from the stressed fish and the undisturbed controls immediately following exercise. Four fish were removed from all aquaria and placed in a 0.08 g/l solution of metomidate hydrochloride. Metomidate hydrochloride was used as it blocks the handling-related release of cortisol into circulation, minimizing endogenous plasma cortisol variability due to sampling (Small 2003). They were then dried with a paper towel, individually weighed, placed in a 2-ml cryogenic storage tube, and snap frozen in liquid nitrogen. Samples were stored at -20°C until processing.

Statistical analysis

Data were analyzed by using the GLM procedures of SAS (SAS version 9.1, SAS Institute, Cary, North Carolina, USA) for a repeated-measures design with the tank serving as the experimental unit. The model included the main effects of treatment and time (0, 7,

and 14 weeks). When the main effects were significant ($P < 0.05$), least squares means separation was accomplished by the PDIF option of SAS.

Results

Cortisol RIA

Sensitivity, accuracy, precision, reproducibility, and parallelism could not be determined for the PBS method as cortisol levels were not detected in any of the extracted samples. The standard curve for the cortisol RIA covered the range of 5.0–200.0 ng/ml. Cortisol concentrations of serially diluted lipid extracts for the CAL and VO methods appeared parallel to the standard curve (data not shown).

Sensitivity of the CAL and VO methods were 4.9 and 6.2 ng/g BW of fish, respectively. After spiking samples with exogenous cortisol, accuracy of the CAL method averaged 100.4% while accuracy averaged 86.7% for the VO method (Table 1). Precision (intra-assay CV) and reproducibility (inter-assay CV) for elevated cortisol levels were 7.3 and 9.6% for CAL, respectively, and 8.2 and 10.6% for VO, respectively. The mean recovery of diluted samples for the CAL and VO methods were 98.4 and 92.1%, respectively.

Fish weight and whole-body cortisol

The weights of exercised fish were statistically similar to controls after 2 weeks of daily exercising (0.37 ± 0.02 g vs. 0.75 ± 0.17 g). Whole-body cortisol concentrations for 0, 7, and 14 days are listed in Fig. 1. Whole-body cortisol increased on day 0 in exercised fish ($P < 0.01$) compared to controls. Whole-body cortisol levels returned to control levels after daily exercise for 1 and 2 weeks, respectively.

Discussion

The purpose of this study was to develop a procedure in which cortisol from catfish fry could be quantified. Methodologies of existing extraction procedures vary and validation of the procedures are not always complete. In the current study, three volume-enhancement methods were examined that increased the

Table 1 Recovery (mean \pm SE, $n = 4$) of exogenous cortisol added to whole-body channel catfish lipid extract

	Cortisol (ng/ml)	CAL method (ng/ml)	Recovery (%)	VO method (ng/ml)	Recovery (%)
10	11.2 \pm 1.2	112.0	8.1 \pm 1.5	81.0	
50	48.7 \pm 1.5	97.4	46.2 \pm 2.1	92.4	
100	96.6 \pm 2.8	96.6	85.3 \pm 5.3	83.5	
200	191.3 \pm 3.2	95.7	180.0 \pm 6.7	90.0	

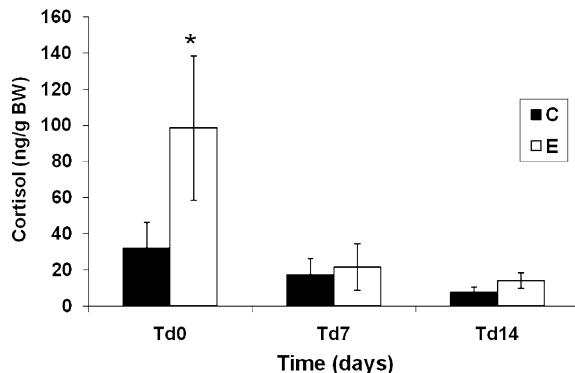


Fig. 1 Whole-body cortisol measurements from fish at 0, 7, and 14 days. C denotes control fish that were not exercised. E denotes fish that underwent intense exercise for 10 min each day. * $P < 0.01$ denotes difference between treatments

volume of the extract. In the CAL method, lipid extract was reconstituted with 100 μ l of zero calibrator A. We hypothesized zero calibrator A would be the most appropriate diluent to reconstitute the lipid extracts with since this is the same buffer used in the cortisol standards. In the PBS method, lipid extract was reconstituted with 1 ml of PBS (PBS method) to increase volume of the sample, as described by Sink et al. (2007) and Ramsay et al. (2006). In the VO method, 100 μ l of vegetable oil per gram of body weight was added to increase the volume of the extract before adding the homogenized sample and ethyl ether, as described in Sink et al. (2007).

Sensitivity, accuracy, precision, reproducibility, and parallelism could not be determined for the PBS method since cortisol levels were not detected in any of the extracted samples. Sink et al. (2007) was able detect cortisol in whole-body lipid extracts obtained from golden shiners, but the PBS method failed the precision and parallelism tests. In the current study, we did not test whether cortisol may have been detected after adding only 100 μ l of PBS to increase the concentration of extracted samples. It is possible that cortisol would have been detected with the addition of only 100 μ l of PBS; however, adding

1 ml of PBS to extracted samples has been previously described for other species of fish (Ramsay et al. 2006; Sink et al. 2007). It is likely that adding 1 ml of PBS diluted the catfish lipid extract too much or the solution of PBS and lipid extract did not emulsify completely and the sample was lost as oil droplets on the side of the tube. Sink et al. (2007) also observed oil droplets on the side of the tube. Results from the current study suggest PBS (1 ml) is not an acceptable method to enhance the volume of the lipid extract used to measure whole-body concentrations of cortisol in channel catfish fry. Furthermore, PBS would not be the most appropriate sample diluent since cortisol standards use zero calibrator A diluent.

Based on the sensitivity, accuracy, precision, reproducibility, and parallelism results, we concluded that the CAL method was the best volume-enhancement method for whole-body cortisol extraction. We tested the CAL method using catfish fry that were exercised for 10 min daily for 2 weeks. Whole-body cortisol levels were higher in stressed fish after 1 day of exercise. However, continued daily exercise did not result in increased cortisol levels after 1 and 2 weeks, respectively. Fry weight was statistically similar in exercised fish compared to control fish by the end of the 2-week study. Repeated stress has also shown habituated reductions in post-stress levels of plasma cortisol in Eurasian perch and rainbow trout (Barton et al. 1987; Jentoft et al. 2005). The effects of repeated stress on growth are not as clear. For example, repeated stress has been shown to be associated with lower growth performance in both the non-domesticated Eurasian perch and the domesticated rainbow trout (Jentoft et al. 2005). However, no difference in growth was observed in juvenile rainbow trout that were repeatedly stressed (Barton et al. 1987). The differences in results obtained among studies may be explained by species differences or differences in the length of time the fish were repeatedly stressed.

These data indicate the CAL method is a valid technique for determining whole-body cortisol in

catfish fry. This procedure will be useful to evaluate stress responses in catfish fry and may lead to improved management practices that minimize stress and improve production efficiency.

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